

Prostaglandin E₂ Regulates Liver versus Pancreas Cell-Fate Decisions and Endodermal Outgrowth

Sahar Nissim,^{1,2,5} Richard I. Sherwood,² Julia Wucherpfennig,² Diane Saunders,² James M. Harris,³ Virginie Esain,³ Kelli J. Carroll,³ Gregory M. Frechette,³ Andrew J. Kim,² Katie L. Hwang,² Claire C. Cutting,² Susanna Elledge,² Trista E. North,^{3,4,*} and Wolfram Goessling^{1,2,4,5,6,*}

¹Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

²Genetics Division, Brigham and Women's Hospital, Boston, MA 02115, USA

³Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA

⁴Harvard Stem Cell Institute, Cambridge, MA 02138, USA

⁵Dana-Farber Cancer Institute, Boston, MA 02115, USA

⁶Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

*Correspondence: [tnorth@bidmc.harvard.edu](mailto:north@bidmc.harvard.edu) (T.E.N.), wgoessling@partners.org (W.G.)

<http://dx.doi.org/10.1016/j.devcel.2014.01.006>

SUMMARY

The liver and pancreas arise from common endodermal progenitors. How these distinct cell fates are specified is poorly understood. Here we describe prostaglandin E₂ (PGE₂) as a regulator of endodermal fate specification during development. Modulating PGE₂ activity has opposing effects on liver versus pancreas specification in zebrafish embryos as well as mouse endodermal progenitors. The PGE₂ synthetic enzyme *cox2a* and receptor *ep2a* are patterned such that cells closest to PGE₂ synthesis acquire a liver fate, whereas more distant cells acquire a pancreas fate. PGE₂ interacts with the *bmp2b* pathway to regulate fate specification. At later stages of development, PGE₂ acting via the *ep4a* receptor promotes outgrowth of both the liver and pancreas. PGE₂ remains important for adult organ growth, as it modulates liver regeneration. This work provides in vivo evidence that PGE₂ may act as a morphogen to regulate cell-fate decisions and outgrowth of the embryonic endodermal anlagen.

INTRODUCTION

How multiple different cell types are generated from common progenitors is a central question in developmental biology. Understanding this process will have therapeutic significance for repair of adult organs. One such context is the development of distinct organs such as the liver, pancreas, lungs, and intestine from the primordial gut endoderm. The specification and outgrowth of these organs are regulated by a dynamic array of signals including fibroblast growth factors (Fgfs), bone morphogenetic proteins (Bmps), retinoic acid, and sonic hedgehog (Zaret and Grompe, 2008). Here we uncover the prostaglandin pathway as a regulator of the specification and outgrowth of the embryonic liver and pancreas.

Whereas the adult organs are histologically and functionally distinct, the embryonic liver and pancreas are thought to arise from a common population of endodermal cells. Experimental support for this bipotential population comes from mouse explant studies, as well as zebrafish cell-lineage studies. Endoderm explants from mouse embryos have the potential to give rise to either liver or pancreatic progenitors depending on extrinsic cues from adjacent mesoderm: Fgfs from the cardiac mesoderm (Deutsch et al., 2001) or Bmps from the septum transversum mesenchyme (Rossi et al., 2001) induce hepatic differentiation, whereas inhibition of these signals causes expression of the pancreatic marker *Pdx1* (Deutsch et al., 2001; Rossi et al., 2001). Similarly, reciprocal effects on hepatic versus pancreatic progenitor populations have been observed in response to Wnt signaling during zebrafish development (Goessling et al., 2008). These reciprocal effects of Fgf, Bmp, and Wnt signals on liver and pancreas progenitor populations suggest that these signals act on common endodermal progenitors to specify one fate at the cost of the other. Indeed, a population of bipotential progenitors has been delineated by single-cell-lineage labeling in embryonic mouse (Miki et al., 2012; Spence et al., 2009; Tremblay and Zaret, 2005) and zebrafish (Chung et al., 2008). In mouse, single-cell labeling of anterior endoderm at early somite stages, prior to expression of organ-specific markers, identified bipotential progenitors as well as closely interspersed ventral pancreas and liver progenitors (Miki et al., 2012; Tremblay and Zaret, 2005). Although these studies map bipotential progenitors and the timing of their segregation into a liver or pancreas anlagen, it is thought that the fates of these segregated populations might be reversible in early somite stages such that modulation of extrinsic signals can shift one fate into another (Miki et al., 2012). In zebrafish, endodermal cells capable of both a liver and exocrine pancreas fate have been identified at the six- to eight-somite stage (Chung et al., 2008). Although these labeling studies localize bipotential progenitors, how this population spatially relates to multiple signals that regulate liver versus pancreas specification is poorly understood.

Prostaglandin molecules have long been recognized as lipid-derived cytokines that modulate diverse biologic processes including vasoregulation, inflammation, and pain (Funk, 2001). In addition, we recently described prostaglandin E₂ (PGE₂) as a

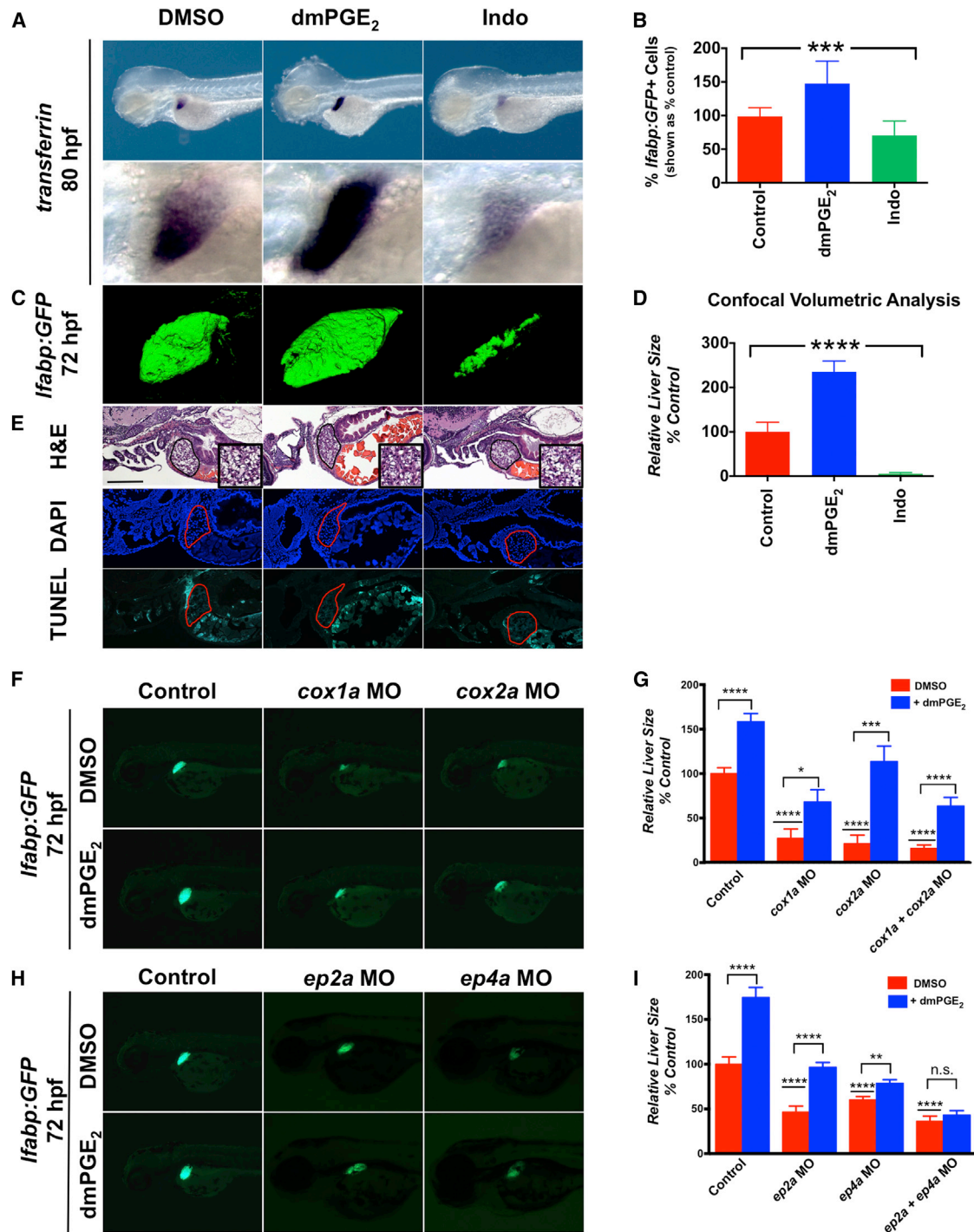


Figure 1. PGE₂ Affects Embryonic Liver Outgrowth

(A) In situ hybridization for *transferrin*, a liver-specific marker, reveals that embryonic liver size is regulated by PGE₂. Exposure to dmPGE₂ from 48 to 80 hpf dramatically increases embryonic liver size (51.4% embryos with an enlarged liver; n = 18/35), whereas exposure to Indo decreases embryonic liver size (76.5% embryos with a smaller liver; n = 26/34). Left lateral view; row below is magnified view.

(B) FACS quantification of GFP⁺ liver cells in *Ifabp:GFP* embryos at 72 hpf following exposure to dmPGE₂ or Indo. dmPGE₂ increases the relative number of liver cells, whereas Indo decreases the relative number of liver cells. (Data are represented as mean ± SEM; ***significant across treatment groups, ANOVA, p = 0.0009.)

(C) Confocal microscopy visualization of the liver in *Ifabp:GFP* embryos at 72 hpf following exposure to dmPGE₂ or Indo. Exposure to dmPGE₂ from 48 to 72 hpf enlarges the developing liver, whereas exposure to Indo severely abrogates liver development. Left lateral view. (Data are represented as mean ± SEM; n = 5 per treatment.)

(legend continued on next page)

conserved regulator of hematopoietic stem cell formation and function (North et al., 2007). However, a role for prostaglandins in developmental fate decisions and solid organ development has not been appreciated, in part because *in vivo* effects cannot be easily studied independent of maternal prostaglandins in mammalian models. Likewise, there is little knowledge of the spatiotemporal expression pattern of components of the prostaglandin pathway during development. In particular, despite a wide body of knowledge of prostaglandin function in the adult gastrointestinal tract, induction and function of components of the prostaglandin pathway in relation to organogenesis of solid endodermal tissues have not been described.

Here we demonstrate a role for PGE₂ in regulating the outgrowth of the liver and pancreas buds *in vivo* as they emerge from the zebrafish gut endoderm. Moreover, we uncover an earlier role for PGE₂ in specification of endoderm into liver versus pancreas progenitors. PGE₂ likewise promotes a liver fate at the cost of a pancreas fate in bipotential mouse embryonic endoderm, suggesting an evolutionarily conserved developmental role for PGE₂. Similar to other canonical pathways regulating organ development, we uncover exquisite spatial and temporal patterning of PGE₂ pathway components consistent with a role in specifying bipotential endodermal cells. Finally, we show that PGE₂ modulates liver regeneration in adult zebrafish, demonstrating that the role of PGE₂ in endodermal outgrowth might have implications for regeneration of adult endodermal derivatives. Together, this work reveals a role for a lipid-derived signaling molecule with morphogenetic properties that regulate endodermal fate decisions and proliferation during development.

RESULTS

Prostaglandin Levels Affect Liver Development

The prostaglandin pathway was identified as a modifier of endodermal organ development through a chemical genetic screen in zebrafish (Garnaas et al., 2012). Specifically, modulators of PGE₂ synthesis and signaling affected embryonic liver size: a larger liver resulted from exposure to compounds that increase prostaglandin signaling activity, whereas a smaller liver size was caused by compounds that decrease prostaglandin signaling. To corroborate the screening results, we exposed zebrafish embryos from 48 to 80 hours postfertilization (hpf) to a long-acting derivative, 16,16-dimethyl-PGE₂ (dmPGE₂), or the nonselective Cox inhibitor indomethacin (Indo), which has been shown to suppress PGE₂ production in zebrafish by mass spectrometry (North et al., 2007). Exposure to dmPGE₂ caused a striking increase in embryonic liver size (51.4% embryos with larger liver;

n = 18/35) as visualized by expression of *transferrin* at 80 hpf (Figure 1A). A larger embryonic liver developed in the presence of Cay10397, an inhibitor of 15-hydroxyprostaglandin dehydrogenase (pgdh) that deactivates prostaglandins *in vivo* and augments endogenous prostaglandin levels (Figure S1E available online). In contrast, exposure to Indo caused a dramatic decrease in embryonic liver size (76.5% embryos with smaller liver; *n* = 26/34), indicating a role for endogenous prostaglandin activity in normal liver development (Figure 1A). Similarly, exposure to the selective Cox1 inhibitor SC-560 or the selective Cox2 inhibitor NS-398 both resulted in a smaller embryonic liver (Figure S1E). The same effects were observed by visualizing fluorescence after treatment of liver fatty acid binding protein:GFP (*lfabp*:GFP) liver reporter fish (Figures S1A and S1F), and these effects were statistically significant by volumetric analysis of confocal images (Figures 1C and 1D). Fluorescence-activated cell sorting (FACS) analysis of these embryos demonstrated statistically significant changes in GFP⁺ liver cell number, but no change in cell size or shape (Figure 1B). Hepatocyte number was increased without changes in cell density or tissue architecture following dmPGE₂ exposure, as revealed by histological analysis (Figure 1E; Figures S1B–S1D). Furthermore, visualization of apoptotic cells in treated embryos showed no significant differences in cell death that could explain effects on organ size (Figure 1E). Visualization of mitotic figures marked by phosphohistone H3 (pHH3⁺) in the liver bud of *lfabp*:GFP reporter fish demonstrated an increase in cell proliferation following exposure to dmPGE₂ and a decrease in cell proliferation following exposure to Indo, suggesting that the effect of PGE₂ is in part mediated by regulating proliferation (Figures S5D and S5E).

The impact of PGE₂ was confirmed by modulation of the prostaglandin synthetic pathway. Morpholino-mediated knockdown of PGE₂ synthesis enzymes *cox1a*, *cox2a*, and *prostaglandin H synthase* (*pges*) resulted in a smaller embryonic liver as quantified by measuring liver size in *lfabp*:GFP embryos at 72 hpf, and this effect was partially rescued by exposure to dmPGE₂ (Figures 1F and 1G; Figure S1G). Likewise, selective pharmacologic blockade of Cox1 and Cox2 by SC-560 and NS-398, respectively, both resulted in a smaller embryonic liver, and this effect was partially rescued by PGE₂ (Figures S1E and S1F). In contrast, knockdown or pharmacologic blockade of pgdh led to a larger embryonic liver, which was further augmented with exogenous dmPGE₂ (Figures S1E–S1G).

The effects of PGE₂ are mediated via the G-protein-coupled receptors EP2 and EP4 (Funk, 2001). We therefore tested whether knockdown of these receptors could generate the same phenotypic effects as reducing levels of PGE₂. Indeed,

(D) Analysis of liver [volume] × [mean fluorescence intensity] visualized by confocal microscopy, corroborating the effect of dmPGE₂ to increase embryonic liver size and Indo to diminish embryonic liver size. (Data are represented as mean ± SEM; *n* = 5 per treatment; ****significant across treatment groups, ANOVA, *p* < 0.0001.)

(E) Histological analysis reveals that modulation of PGE₂ levels changes embryonic liver size but does not alter liver morphology (*n* = 5 per treatment; magnified views are shown in the insets). TUNEL analysis shows no apoptosis following modulation of PGE₂ activity (*n* = 5 per treatment). The scale bar represents 100 μm. (F–I) Morpholino-mediated knockdown of PGE₂ synthetic enzymes (F and G) or PGE₂ receptors *ep2a* and *ep4a* (H and I) alters liver development, as visualized by fluorescence in *lfabp*:GFP embryos. Left lateral view. Impact on liver size is quantified by [area] × [fluorescence intensity] in *lfabp*:GFP embryos as shown in (G) and (I). (Data are represented as mean ± SEM; *n* ≥ 30 per condition; significant by *t* test comparing each morpholino [MO] versus control and each morpholino without versus with dmPGE₂; *****p* < 0.0001, **p* < 0.05, ***p* < 0.01, ****p* < 0.001; n.s., not significant.)

See also Figure S1.

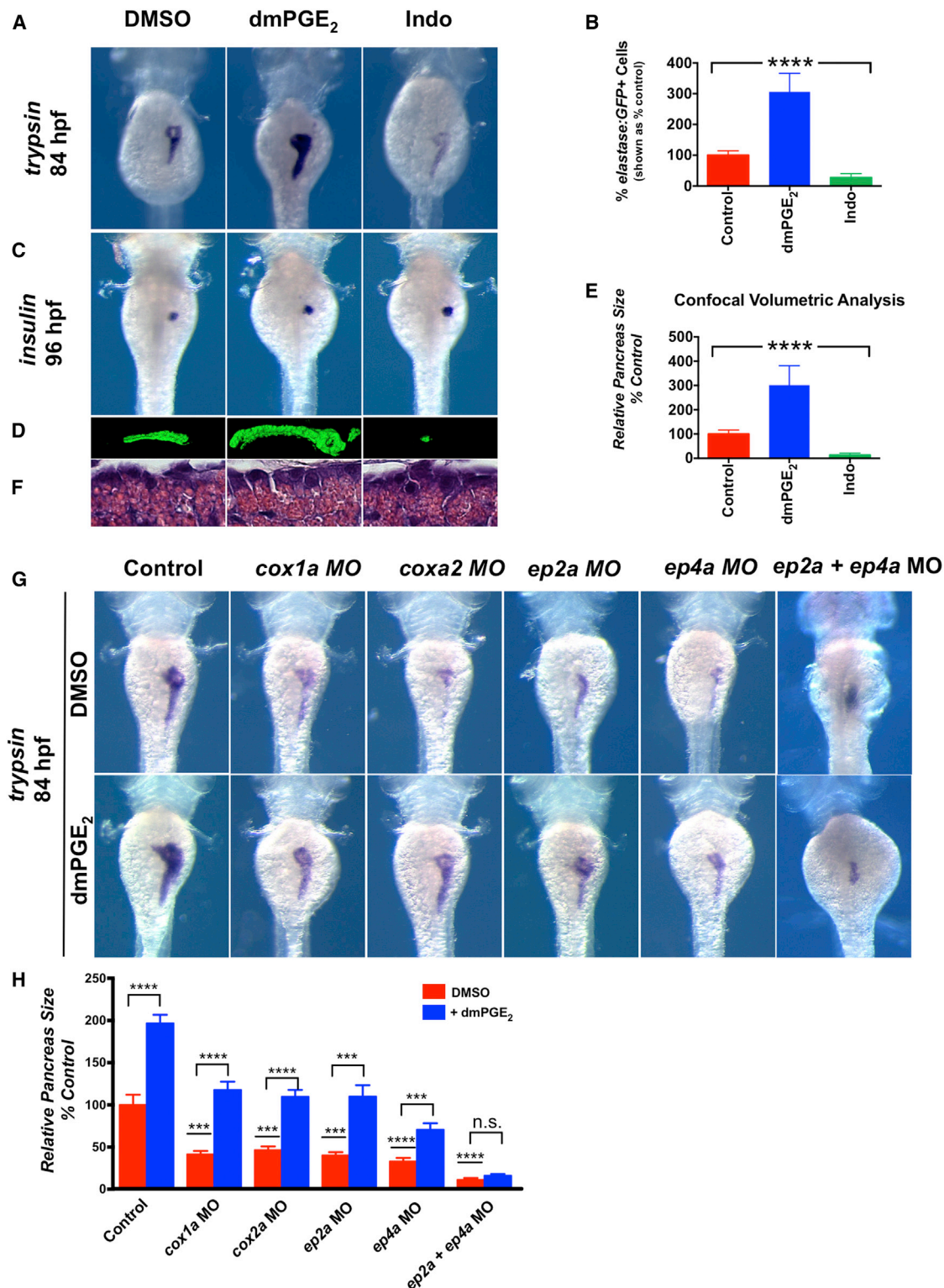


Figure 2. PGE₂ Affects Embryonic Pancreas Outgrowth

(A) In situ hybridization for *trypsin*, an exocrine pancreas-specific marker, following exposure to dmPGE₂ or Indo reveals that embryonic pancreas size is regulated by PGE₂. dmPGE₂ increases exocrine pancreas size (57.6% embryos with an enlarged pancreas; n = 38/66), whereas Indo diminishes exocrine pancreas size (70.6% embryos with a smaller pancreas; n = 36/51). Dorsal view.

(B) FACS quantification of GFP⁺ exocrine pancreas cells in *elastase:GFP* embryos at 96 hpf following exposure to dmPGE₂ or Indo. dmPGE₂ increases and Indo decreases the relative number of exocrine pancreas cells. (Data are represented as mean ± SEM; ****significant across treatment groups, ANOVA, p < 0.0001.)

(legend continued on next page)

morpholino-mediated knockdown of either *ep2a* or *ep4a* resulted in a smaller embryonic liver, and this effect was only partially rescued by exogenous addition of PGE₂ (Figures 1H and 1I). Moreover, combined knockdown of both the *ep2a* and *ep4a* receptors more severely abrogated liver development (Figure 1I; Figure S1G). Whereas the combined knockdown of both *cox1a* and *cox2a* synthetic enzymes could be partially rescued by exogenous dmPGE₂ (Figure 1G; Figure S1G), the combined knockdown of both *ep2a* and *ep4a* receptors could not be rescued by exogenous dmPGE₂ (Figure 1I; Figure S1G), consistent with PGE₂ acting through these receptors to enlarge the developing liver and further confirming the role of PGE₂ signaling. These data provide in vivo demonstration that PGE₂ activity modulates embryonic liver development.

Signaling through EP2 and EP4 is thought to regulate phosphorylation by increasing intracellular levels of cAMP and activating the downstream effector kinase PKA (Funk, 2001). Therefore, to test whether PGE₂ acts through these downstream effectors to promote liver outgrowth, zebrafish embryos were exposed to forskolin, a cAMP activator, or H89, a PKA inhibitor, from 48 to 72 hpf. Augmenting cAMP activity resulted in a larger embryonic liver, whereas inhibiting PKA activity resulted in a smaller embryonic liver (Figure S1H). Furthermore, the effect of dmPGE₂ to augment liver size was blunted by H89, whereas the effect of Indo to diminish liver size was countered by forskolin (Figure S1H). These data are consistent with PGE₂ acting through downstream effectors cAMP and PKA to promote liver outgrowth.

Prostaglandin Levels Affect Pancreas Development

Given the shared origin of liver and pancreas from gut endoderm, we next examined the impact of PGE₂ on pancreas development. Zebrafish embryos were exposed to dmPGE₂ or Indo during outgrowth of the exocrine pancreas from 48 to 84 hpf and examined for markers of exocrine (*trypsin*, *carboxypeptidase A*) or endocrine (*insulin*) differentiation. dmPGE₂ caused a dramatic increase in exocrine pancreas size, whereas Indo diminished exocrine pancreas size or completely abrogated exocrine marker expression (Figure 2A; Figure S2A). A statistically significant effect was confirmed by volumetric analysis of the exocrine pancreas as visualized by confocal microscopy of transgenic *trypsin:GFP* fish (Figures 2D and 2E). FACS analysis of *elastase:GFP* reporter fish demonstrated statistically significant changes in GFP⁺ exocrine pancreas cell number but no change in cell size or shape (Figure 2B). In contrast, modulation of PGE₂ levels had no observable effects on endocrine pancreas forma-

tion as visualized by *insulin* expression (Figure 2C). No histological differences in the exocrine pancreas were detectable in all treated embryos (Figure 2F; Figure S2C) nor discernable effects on cell death as assessed by TUNEL staining (Figure S2B).

We next tested whether inhibition of PGE₂ synthesis enzymes or receptors would similarly influence exocrine pancreas development. Knockdown of *cox1a*, *cox2a*, or *pges* or exposure to SC-560 or NS-398 abrogated development of the exocrine pancreas visualized by expression of *trypsin* at 84 hpf, and this effect was partially rescued by exogenous dmPGE₂ (Figures 2G and 2H; Figure S2E). Knockdown of either *ep2a* or *ep4a* resulted in a smaller exocrine pancreas, and this effect could only be partially rescued by exogenous dmPGE₂ (Figures 2G and 2H). Again, combined knockdown of both receptors *ep2a* and *ep4a* caused severe diminution of the developing exocrine pancreas, and this effect could no longer be rescued by dmPGE₂, consistent with PGE₂ acting through these receptors to enlarge the developing pancreas (Figures 2G and 2H). Inhibition of PGE₂ breakdown by the *pgdh* inhibitor Cay10397 augmented *trypsin* expression, demonstrating that, similar to the liver bud, increasing endogenous PGE₂ levels results in a larger exocrine pancreas (Figure S2D). These data provide in vivo evidence that levels of PGE₂ modulate embryonic pancreas development. Further experiments using dmPGE₂ and Indo and expression analysis of the intestinal marker *intestinal fatty acid binding protein (ifabp)* demonstrated an impact on intestinal development at 96 hpf (Figures S2F and S2G). These observations suggest that PGE₂ activity might regulate the outgrowth of other endodermal derivatives in addition to the liver and pancreas.

Prostaglandin Has Reciprocal Effects on Liver versus Pancreas Endodermal Specification

We have shown that prostaglandin activity is required for normal development of the liver and pancreas and that augmenting prostaglandin levels enlarges both the developing liver and pancreas. These effects were observed when zebrafish embryos were treated after 48 hpf, when liver and pancreas populations have begun to express markers of mature differentiation. We next sought to elucidate whether PGE₂ had a similar role before 24 hpf, a period when markers of specified liver and pancreas progenitors are first expressed but before differentiation and expansion of these progenitors into specialized populations. Zebrafish embryos were exposed to dmPGE₂ at various time frames before and after 24 hpf. Surprisingly, exposure between 12 and 20 hpf resulted in statistically significant opposing effects

(C) In situ hybridization for *insulin* following exposure to dmPGE₂ or Indo (n > 30 per condition). Dorsal view.

(D) Confocal microscopy visualization of exocrine pancreas in *trypsin:GFP* embryos at 96 hpf following exposure to dmPGE₂ or Indo. Exposure to dmPGE₂ enlarges and Indo severely diminishes pancreas development (n = 5 per condition). Right lateral view.

(E) Analysis of pancreas [volume] × [mean fluorescence intensity] visualized by confocal microscopy, corroborating the effect of dmPGE₂ to increase and Indo to diminish embryonic pancreas size. (Data are represented as mean ± SEM; ****significant across treatment groups, ANOVA, p < 0.0001.)

(F) Histological analysis reveals that modulating PGE₂ levels changes embryonic pancreas size without discernible changes in pancreas cell morphology (n = 5 per treatment). Shown are exocrine pancreas cells with apical accumulation of secretory granules.

(G and H) Morpholino-mediated knockdown of PGE₂ synthetic enzymes or PGE₂ receptors *ep2a* and *ep4a* abrogates pancreas development, and this effect is partially rescued by addition of dmPGE₂, as visualized by in situ hybridization for *trypsin* expression. Combined knockdown of both *ep2a* and *ep4a* receptors severely diminishes pancreas development, and this cannot be rescued by addition of dmPGE₂. Left lateral view. Impact on pancreas size is quantified by [area] × [fluorescence intensity] in *trypsin:GFP* fish as shown in (H). (Data are represented as mean ± SEM; n > 30 per condition; significant by t test comparing each morpholino treatment versus control and each morpholino treatment without versus with dmPGE₂, ****p < 0.0001, ***p < 0.001; n.s., not significant.) See also Figure S2.

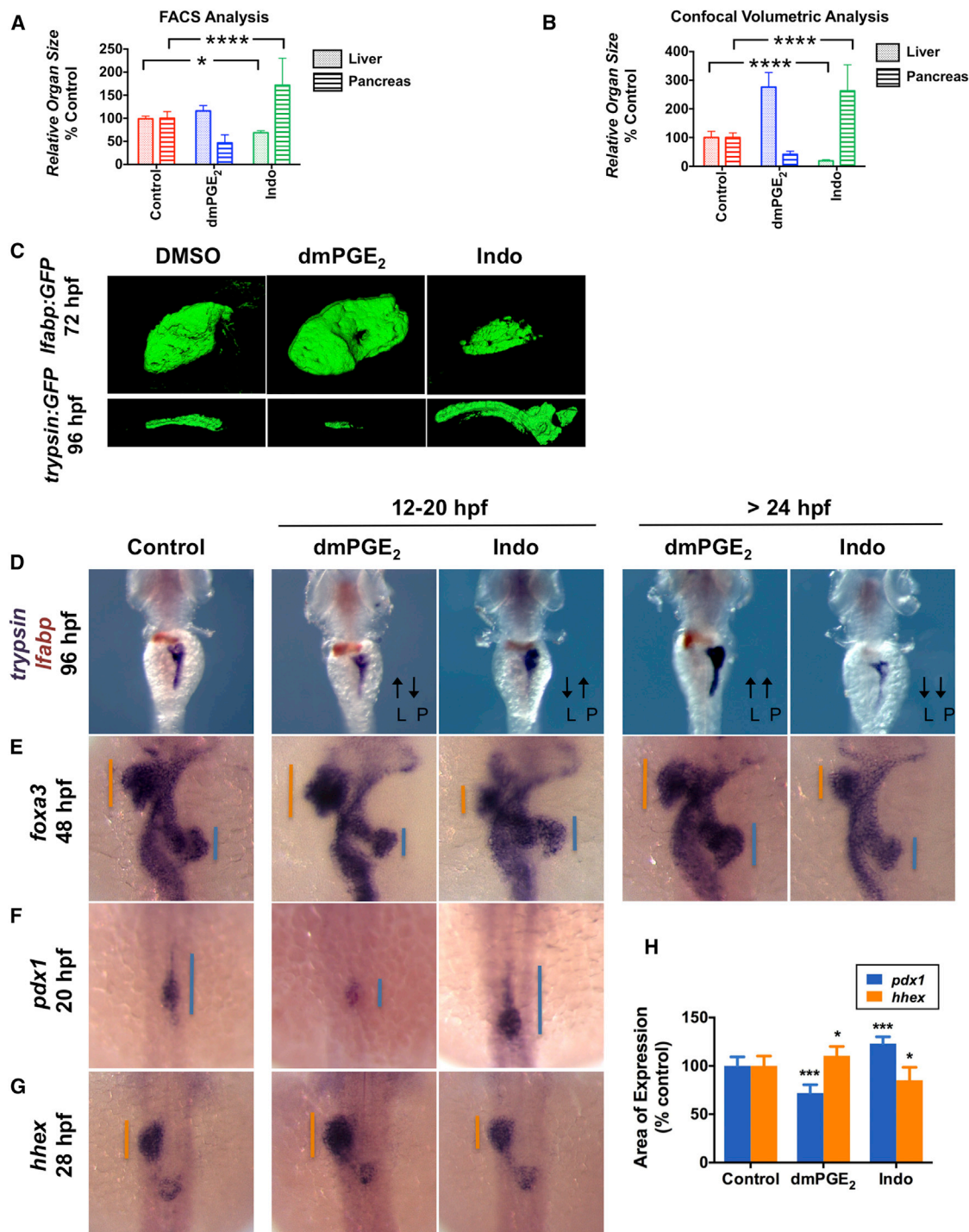


Figure 3. PGE₂ Influences Liver versus Pancreas Specification

(A) FACS quantification of liver and pancreas GFP⁺ cells in *lfabp*:GFP or *elastase*:GFP fish, respectively, at 96 hpf following exposure to dmPGE₂ or Indo from 12 to 20 hpf. (Data are represented as mean ± SEM; significant across treatment groups, ANOVA, ****p < 0.0001, *p < 0.05.)

(B) Analysis of organ [volume] × [fluorescence intensity] of the liver or pancreas in *lfabp*:GFP fish at 72 hpf or *trypsin*:GFP fish at 96 hpf, respectively, visualized by confocal microscopy following treatment with dmPGE₂ or Indo from 12 to 20 hpf. (Data are represented as mean ± SEM; significant across treatment groups, ANOVA, ****p < 0.0001; n = 5 per condition.)

(C) Confocal microscopy of the liver at 72 hpf in *lfabp*:GFP fish or pancreas at 96 hpf in *trypsin*:GFP fish following exposure to dmPGE₂ or Indo from 12 to 20 hpf. Volume and fluorescence intensity are quantified in (B).

(D) Double in situ hybridization for *trypsin* (purple) + *lfabp* (brown) at 96 hpf reveals reciprocal effects on organ size following 12–20 hpf exposure to dmPGE₂ or Indo. Dorsal view. L, liver; P, pancreas.

(legend continued on next page)

on the liver and pancreas, with dmPGE₂ resulting in a larger liver but smaller pancreas and Indo resulting in a smaller liver but larger pancreas, as quantified by FACS analysis of *lfabp:GFP* or *elastase:GFP* reporter fish (Figure 3A) and confocal volumetric analysis (Figures 3B and 3C). The reciprocal effect on liver versus pancreas size when PGE₂ levels are modulated between 12 and 20 hpf contrasts with the effect observed after 24 hpf, when impact on liver and pancreas size is in parallel.

We further explored this reciprocal effect by simultaneously visualizing the emerging liver and pancreas buds marked by the panendodermal marker *foxa3* at 48 hpf as well as markers of differentiated liver and pancreas cells at 96 hpf. Exposure to dmPGE₂ or Indo after 24 hpf confirmed a parallel effect; in contrast, earlier exposure to these compounds at 12–20 hpf had opposing effects on the developing liver and pancreas buds (Figure 3E; Figure S3A). Exposure to dmPGE₂ from 12 to 20 hpf enlarges the liver bud but diminishes the pancreas bud at 48 hpf (Figure 3E), and larger liver but smaller pancreas organs develop by 96 hpf (Figure 3D). Conversely, Indo treatment from 12 to 20 hpf results in smaller liver and larger pancreas buds at 48 hpf (Figure 3E), and smaller liver and but larger pancreas organs are observed at 96 hpf (Figure 3D).

To investigate whether these reciprocal effects on the nascent liver and pancreas organs ensue from effects on their respective progenitor populations, we examined the impact on endodermal progenitors specified to a liver or pancreas fate. dmPGE₂ exposure from 12 to 20 hpf diminished the pancreas progenitor population marked by *pdx1* but augmented liver progenitors visualized with *hhex* (Figures 3F and 3G). Conversely, exposure to Indo from 12 to 20 hpf resulted in a larger pancreas progenitor population but a diminished liver progenitor population (Figures 3F and 3G). Quantification of the area of pancreas progenitors marked by *pdx1* or liver progenitors visualized by *hhex* expression confirmed a statistically significant change in response to modulating PGE₂ activity (Figure 3H). To assess whether the observed effects of PGE₂ activity on the size of the pancreas or liver progenitor populations at 20 or 28 hpf, respectively, are mediated by differential expansion of progenitors, we quantified mitotic figures marked by pHH3 at 18 hpf in *sox17:GFP* endoderm reporter fish. Modulating PGE₂ activity between 12 and 18 hpf results in no significant changes in the number of pHH3⁺ *sox17:GFP*⁺ cells (Figure S5A). These data suggest that the reciprocal effects of early PGE₂ activity on liver versus pancreas development are caused by opposing effects on the specification of progenitor populations.

Put together, these data uncover a biphasic role for PGE₂ levels in liver and pancreas development. At the earlier phase from 12 to 20 hpf before the liver and pancreas buds have emerged, PGE₂ expands the liver progenitor population but

diminishes the pancreas progenitor population. This earlier time frame corresponds to the stage when bipotential progenitors capable of both liver and pancreas fates have been identified (Chung et al., 2008). Therefore, these reciprocal effects raise the possibility that PGE₂ acts on bipotential endodermal progenitors to promote a liver fate at the expense of a pancreas fate.

Prostaglandin Affects Liver versus Pancreas Specification of Murine Bipotential Endodermal Progenitors

To directly test the hypothesis that PGE₂ regulates the specification of bipotential endodermal progenitors toward a liver fate at the cost of a pancreas fate and suggests similar processes across species, we derived a population of bipotential endodermal progenitors through monolayer differentiation of mouse embryonic stem cells. These bipotential progenitors express the hepatopancreatic markers Hnf1β (Haumaitre et al., 2005; Lokmane et al., 2008), Prox1 (Burke and Oliver, 2002), and Onecut1 (Clotman et al., 2002; Jacquemin et al., 2003; Rausa et al., 1997) as well as the pan-endodermal marker FoxA2 (Figure S4). Upon further differentiation, subpopulations of these cells spontaneously express Hnf4α, a marker of hepatic specification, or Pdx1, a marker of pancreatic specification. Manipulation of transforming growth factor β (TGF-β) and Fgf signaling allows enrichment of hepatic or pancreatic specification, whereas both subpopulations arise spontaneously in “neutral” conditions (Figures S4A–S4C). When levels of PGE₂ were increased in neutral conditions by incubation with dmPGE₂ or Cay10397, differentiation into Pdx1⁺ cells decreased, whereas differentiation into Hnf4α⁺ cells increased significantly (Figure 4). In contrast, when levels of PGE₂ were decreased by incubation with Indo, SC-560, or NS-398, the majority of the population was specified into a Pdx1⁺ pancreas fate but specification into a liver fate was dramatically reduced (Figure 4). These effects of PGE₂ modulation occurred in as little as 16 hr, making it unlikely that differential proliferation could explain the changes in specification. Indeed, despite the statistically significant changes in cell differentiation, cell density as quantified by nuclear staining did not change with modulation of prostaglandin activity (Figure 4C). Additionally, we quantified proliferation by the percentage of FoxA2⁺ endoderm cells that were also pHH3⁺ after 18 hr of pharmacologic PGE₂ modulation and found no statistically significant differences across the treatment conditions (Figures S5B and S5C). These results suggest that low levels or absence of PGE₂ promotes pancreas specification, whereas PGE₂ is necessary for liver specification. Furthermore, these results support the hypothesis that PGE₂ regulates bipotential endoderm progenitor differentiation toward a liver versus pancreas fate across vertebrate species.

(E) In situ hybridization for *foxa3* at 48 hpf reveals reciprocal effects on the liver and pancreas buds emerging from the gut endoderm. The extent of the liver bud is indicated in orange and of the pancreas bud in blue. Dorsal view.

(F and G) Early exposure to dmPGE₂ or Indo results in a smaller (89.1% of embryos; n = 41/46) or larger (95.1% of embryos; n = 39/41) domain, respectively, of pancreas progenitor cells visualized by *pdx1* at 20 hpf, and a larger (68.6% of embryos; n = 35/51) or smaller (72.7% of embryos; n = 32/44) domain, respectively, of liver progenitor cells marked by *hhex* at 28 hpf. Dorsal view.

(H) Quantification of the area of pancreas progenitors or liver progenitors following exposure to dmPGE₂ or Indo during somitogenesis, shown as % control. (Data are represented as mean ± SEM; significant across treatment groups, ANOVA, ***p < 0.001, *p = 0.013; n = 5 per treatment group.)

See also Figure S3.

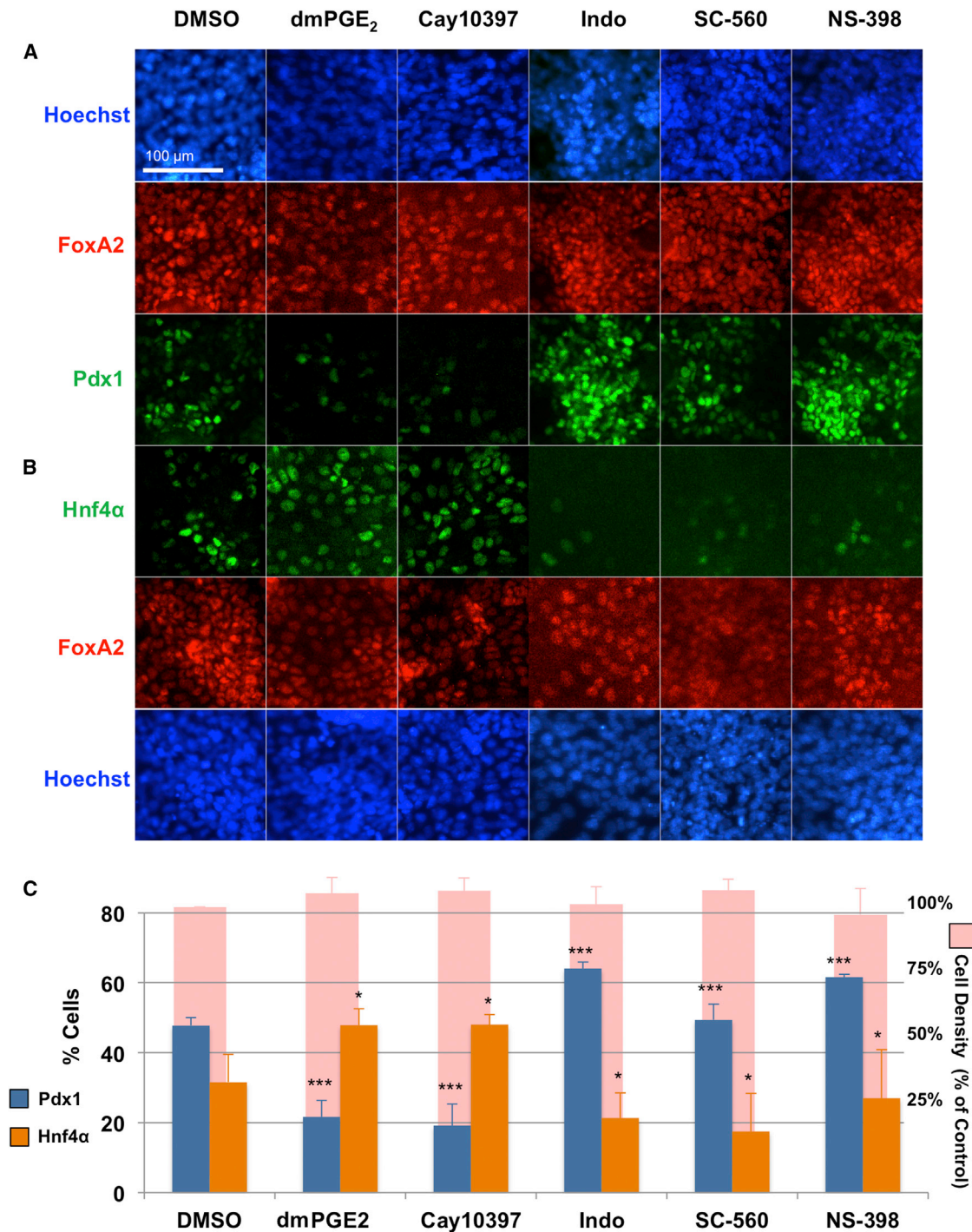


Figure 4. Liver versus Pancreas Specification of Mouse Bipotential Endoderm Progenitors Is Influenced by PGE₂

(A and B) Immunohistochemistry analysis of differentiating mouse endodermal progenitors following 24 hr exposure to various modulators of PGE₂ activity. Panendodermal cells marked by FoxA2 (red) are capable of either pancreas specification marked by Pdx1 (green in A) or liver specification marked by Hnf4α (green in B). The scale bar represents 100 μm.

(C) The percentage of FoxA2⁺ cells that become Pdx1⁺ (blue) or Hnf4α⁺ (orange) following exposure to various modulators of PGE₂ activity. (Data are represented as mean ± SEM; significant compared to DMSO by ANOVA test, ***p < 0.001, *p < 0.05; three independent experiments.) Shown also is cell density as % of control (pink), and differences are not significant by ANOVA test (p = 0.854).

See also [Figures S4](#) and [S5](#).

Prostaglandin Pathway Genes Are Patterned to Regulate Liver versus Pancreas Specification of Endoderm

Having demonstrated *in vivo* and in the context of mouse bipotential endoderm that PGE₂ activity influences liver versus pancreas specification, we next examined expression of components of the prostaglandin pathway in relation to endodermal progenitors. We first examined mammalian expression by microarray analysis in mouse embryonic stem cells at various stages of differentiation, and confirmed that *Ep2* and *Ep4* expression is enriched in a population of bipotential mouse endoderm relative to primitive ectoderm or lateral plate mesoderm (Figure 5A). We further confirmed conserved enrichment of the PGE₂ receptors *ep2a* and *ep4a* in the zebrafish endoderm monolayer by analysis of GFP⁺ cells sorted from a primitive endoderm reporter fish, *sox17:GFP*, at the six- to eight-somite stage. We found that *ep2a* is highly enriched in the zebrafish endoderm, consistent with responsiveness to PGE₂ in this population (Figure 5B). A population of bipotential liver and exocrine pancreas progenitors has been delineated by single-cell labeling in the endoderm monolayer of zebrafish (Chung et al., 2008), located between somites 1 and 3 at the six- to eight-somite stage. We therefore examined expression of components of the prostaglandin pathway in relation to this population of bipotential endodermal cells. At 10 hpf, when somitogenesis is beginning, *ep2a* is expressed in a population of cells adjacent to the notochord, spanning four cells from the midline and localized along the anterior-posterior axis in the region where the first somites will form (Figure S6A). Remarkably, at the six-somite stage (12 hpf), the PGE₂ receptor *ep2a* is localized to endoderm between somites 1 and 3 in a population of cells spanning four cells from the midline (Figure 5C; Figures S6C and S6D), colocalized with the population of bipotential progenitors (Chung et al., 2008). We next examined expression of PGE₂ synthesis components. Expression of *cox2a* was found in a population of cells on the lateral edge of the population expressing *ep2a* (Figure 5C; Figure S6A). Expression of *cox1a* was found in a broader population of cells even more lateral to the *ep2a*-expressing cells (Figure S6B). Expression of other PGE₂ pathway genes including the receptor *ep4a* and the degrading enzyme *pgdh* did not appear to be specifically patterned in relation to the population of bipotential progenitors during early somitogenesis (Figure 5D; Figure S6B). Because *cox2a* is known to be an inducible and rate-limiting enzyme in PGE₂ synthesis, this patterning of *cox2a* expression is consistent with the highest levels of PGE₂ occurring at the most lateral side of the *ep2a*-expressing cells and lower levels of PGE₂ occurring at the most medial aspect of the *ep2a*-expressing cells.

Given the finding that the PGE₂ receptors *ep2a* and *ep4a* are differentially patterned during embryogenesis, we exposed zebrafish embryos to receptor-selective agonists in order to delineate the role of specific receptors at different stages of development. Exposure to Ep1 or Ep3 agonists sulprostone or Ono 248, respectively, had no significant impact on developing pancreas or liver size (Figure 5E). In contrast, exposure to selective Ep2 agonists butaprost or Ono 259 during somitogenesis 12–20 hpf results in a smaller pancreas but larger liver (Figure 5E), similar to dmPGE₂. Moreover, exposure to a selective Ep4 agonist, Ono 329, during somitogenesis has no significant

impact on developing pancreas or liver size (Figure 5E), consistent with expression of *ep2a* but not *ep4a* corresponding to the population of bipotential endodermal progenitors. At later time points during organ outgrowth, we have shown that exposure to dmPGE₂ results in a larger pancreas and liver (Figures 3A–3D). Exposure to the Ep4 agonist Ono 329 at 48–96 hpf results in a larger pancreas and liver organ (Figure 5G). In contrast, no significant effects on developing pancreas or liver size were observed following exposure to selective Ep2 agonists butaprost or Ono 259 (Figure 5G). Consistent with these receptor-specific effects, we find that *ep4a* but not *ep2a* is expressed in the developing liver bud during liver outgrowth (Figure 5F). Put together, these expression patterns and receptor-specific functional data suggest that the specificity and changing roles of PGE₂ through development are dictated by receptor localization.

We have previously demonstrated the importance of developmental signaling pathways for liver regeneration (Goessling et al., 2008). Having shown a significant role for PGE₂ during embryonic liver outgrowth, we examined whether PGE₂ impacts adult liver regeneration. Partial hepatectomies were performed in adult fish, and regrowth was quantified after exposure to PGE₂ modifiers. We found that PGE₂ regulates adult liver regeneration and, similar to embryonic liver outgrowth, this role is predominantly mediated via the *ep4a* receptor (Figures S6E and S6F). Similar to the developmental context of liver bud outgrowth, PGE₂ regulates cell proliferation in the regenerating liver (Figure S6G). These data suggest that the developmental role for PGE₂ in liver outgrowth might be recapitulated in the context of adult liver regeneration.

Prostaglandin and Bmp Signaling Pathways Interact to Regulate Liver versus Pancreas Specification

Much like the impact of prostaglandin described here, Bmp signaling has been shown to promote hepatic versus pancreatic fate in both mouse (Rossi et al., 2001) and zebrafish (Chung et al., 2008) embryonic endoderm. Moreover, similar to *cox2a* at the six- to eight-somite stage, *bmp2b* expression is spatially regulated in two bilateral stripes at the ten-somite stage and affects liver versus pancreas specification (Chung et al., 2008). Given these parallels in both timing and pattern of expression, we explored the possibility that these pathways interact to regulate liver versus pancreas specification. Exposure to Indo from 12 to 20 hpf diminishes *bmp2b* expression in the lateral plate mesoderm at 20 hpf, whereas exposure to dmPGE₂ increases expression of *bmp2b* (Figure 6A). This finding demonstrates that PGE₂ activity can regulate *bmp2b* expression, and therefore suggests that the prostaglandin pathway might be acting via *bmp2b* signaling to regulate liver versus pancreas specification. To further test this possibility, we modulated both prostaglandin activity as well as *bmp2b* activity and assayed the impact on pancreas specification marked by *pdx1*. As previously reported, activation of *bmp2b* in heat-shock-inducible *hs:bmp2b* transgenic fish diminishes the pancreas progenitor population marked by *pdx1* (Chung et al., 2008; Figures 6B and 6C). In contrast, inhibition of bmp signaling by exposure to dorsomorphin, an inhibitor of bmp receptors alk 2, 3, and 6, or by activation of a dominant-negative bmp receptor in heat-shock-inducible *hs:dnBmpR* transgenic fish dramatically promotes the pancreas progenitor population (Figures 6B and 6C). In support of a

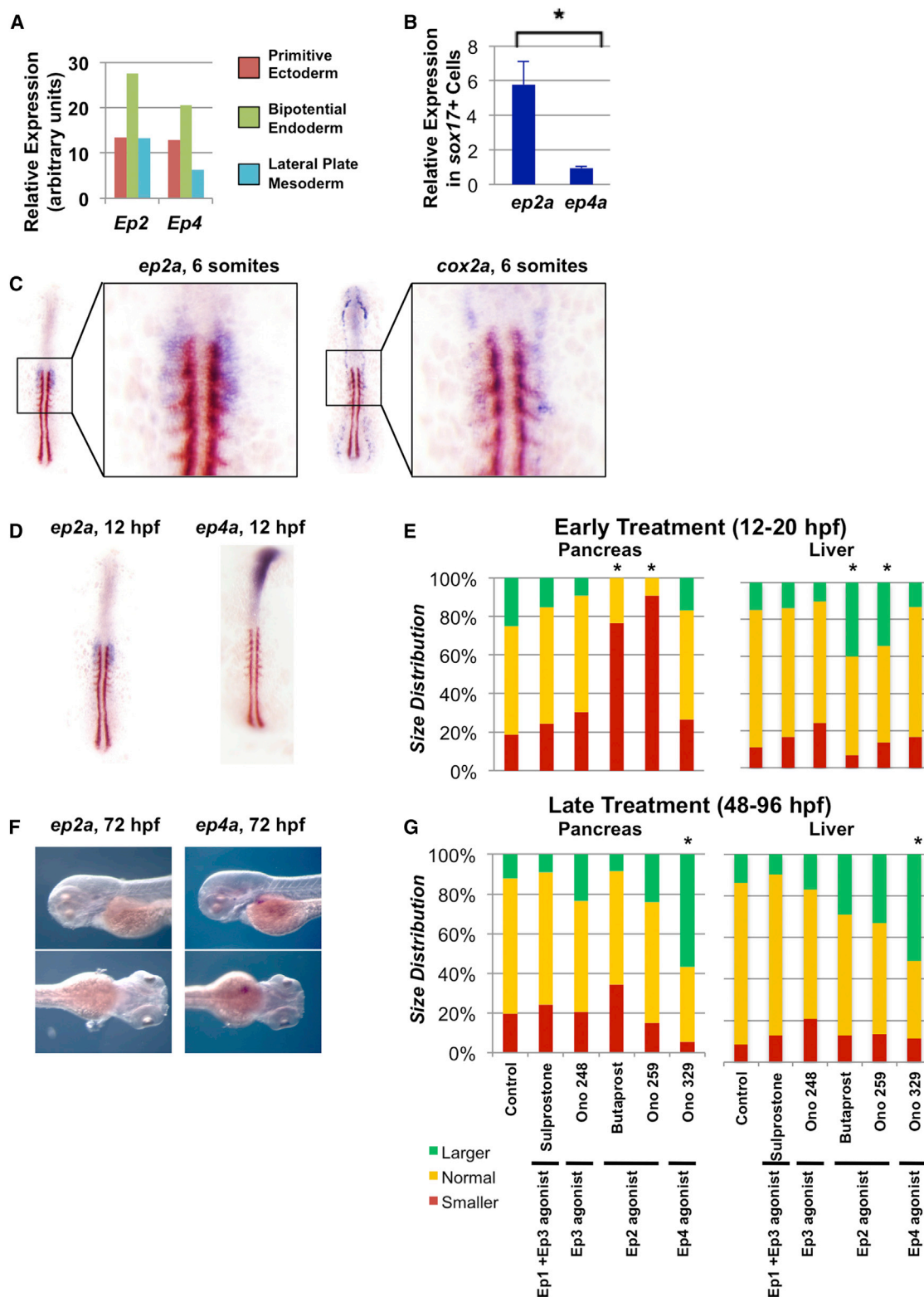


Figure 5. Prostaglandin Pathway Genes Are Patterned to Regulate Endoderm Specification

(A) Microarray analysis reveals enrichment of *Ep2* and *Ep4* in bipotential endoderm progenitors compared to primitive ectoderm or lateral plate mesoderm derived from mouse embryonic stem cells, shown in arbitrary units.

(B) Quantitative PCR analysis of *sox17*⁺ endodermal cells sorted from *sox17:GFP* zebrafish embryos reveals enrichment of *ep2a* compared to *ep4a*. (Data represented as mean \pm SEM; t test, *p < 0.05; n = 3.)

(legend continued on next page)

signaling cascade in which *bmp2b* acts downstream of the prostaglandin pathway, the effect of dmPGE₂ to promote pancreas progenitors is blunted by *bmp* receptor blockade with dorsomorphin or heat-shock induction of *dnBmpR* (Figures 6B and 6C). Conversely, the effect of Indo to promote pancreas progenitors is blunted following activation of *bmp2b* (Figures 6B and 6C). These data support a model in which the prostaglandin and *bmp2b* pathways interact to regulate liver versus pancreas specification, and that PGE₂ acts at least in part by positively regulating *bmp2b* expression.

DISCUSSION

In this study, we uncover evidence of a role for PGE₂ in embryonic development. Through genetic and chemical approaches in zebrafish embryos, we provide in vivo demonstration that PGE₂ activity has a critical role in the specification and outgrowth of the liver and pancreas. During early somitogenesis, we reveal that PGE₂ activity regulates specification of endoderm into a liver versus pancreas fate. We further provide evidence suggesting mammalian conservation of this role in the context of mouse embryonic endoderm. Later in embryonic development, we uncover a role for PGE₂ activity in regulating outgrowth of the liver and pancreas buds. In line with this role in regulating embryonic liver outgrowth, PGE₂ activity influences liver regeneration in the adult zebrafish.

PGE₂ Regulates the Fate Specification of Bipotential Endoderm

We show that PGE₂ activity promotes a liver progenitor population at the cost of a pancreas progenitor population. These findings are consistent with a role for PGE₂ in regulating cell-fate decisions in a population of bipotential endodermal progenitors. An alternative interpretation is that PGE₂ activity might differentially influence the proliferation or maintenance of endodermal cells already specified to a liver or pancreas fate. In support of the first model, the reciprocal effects of prostaglandin activity occur at a stage when bipotential endodermal cells have been delineated (Chung et al., 2008). Moreover, we examine a population of mouse endodermal cells that can be specified into both liver and pancreas fates, and show that modulating prostaglandin activity can shift this population predominantly into one fate or the other. We show that in both the in vivo context of zebrafish endoderm as well as the in vitro context of mouse bipotential endodermal cells, modulating prostaglandin activity does not differentially alter cell proliferation. Put together, these observations argue that PGE₂ acts on bipotential endodermal cells to guide specification toward a liver fate versus a pancreas fate.

In conjunction with our functional data, we reveal exquisite patterning of genes in the prostaglandin signaling pathway consistent with a key role in endodermal specification. The prostaglandin receptor *ep2a* is expressed in a population of endoderm corresponding to the previously characterized bipotential progenitor population (Chung et al., 2008). This pattern raises the intriguing possibility that *ep2a* might serve as an early marker of liver-pancreas bipotential progenitors. Although studies in zebrafish and mouse embryos have characterized the signals directing the specification of this population, a marker for this population has not been described. Our microarray data show enrichment of *Ep2* in mouse bipotential endoderm and suggest that the patterning of *ep2a* in zebrafish might be conserved.

Understanding PGE₂ as a Morphogen

Concomitant with patterning of *ep2a*, the rate-limiting enzyme in PGE₂ synthesis *cox2a* is patterned in two bilateral stripes such that endodermal cells closest to this source of prostaglandin are specified to become liver, whereas endodermal cells farther from this source of prostaglandin are specified to become exocrine pancreas. These findings lay the groundwork for understanding prostaglandin activity as a morphogen whereby a gradient of prostaglandin activity might pattern a field of endoderm (Figure 7). This model makes several predictions. First, although there is no direct way to visualize PGE₂ in vivo, as has been achieved with fluorescent fusion proteins (Müller et al., 2012), we speculate that there might be a graded distribution of the readily diffusible PGE₂ molecule acting on the population of *ep2a*-expressing cells based on the distinct spatial restriction of *cox2a* expression. A morphogen gradient of another nonpeptidic signaling molecule, retinoic acid, has long been functionally recognized but only recently was visually proven (Shimozono et al., 2013). Prostaglandins are widely assumed to act as paracrine hormones exerting effects by interacting with extracellular receptors. However, although prostaglandin transport proteins have been identified in the cell membrane, the mechanisms regulating prostaglandin exit from cells and how far they act are poorly understood (Schuster, 2002). Second, a prevailing view of morphogen gradients postulates that uniform clearance across the exposed tissue generates a decaying concentration gradient of the morphogen (Rogers and Schier, 2011). Consistent with this notion, expression of the PGE₂-degrading enzyme *pgdh* is uniform. Moreover, we show that targeted knockdown or pharmacologic inhibition of this enzyme activity has phenotypic consequences similar to augmentation of PGE₂ levels. Third, consistent with a morphogen model, our observations in mouse bipotential endoderm suggest that this cell population has different genetic responses to different levels of PGE₂. High levels

(C) Double in situ hybridization for *ep2a* (purple) or *cox2a* (purple) and *myod* (brown) in zebrafish embryos at the six-somite stage.

(D) Double in situ hybridization for *ep2a* (purple) or *ep4a* (purple) and *myod* (brown) in zebrafish embryos at the six-somite stage.

(E) Size distribution of pancreas or liver progenitor domain following exposure to pharmacologic agonists or antagonists of various PGE₂ receptors from 12 to 20 hpf. Impact on pancreas progenitors was assessed at 20 hpf by in situ hybridization to *pdx1*, and impact on liver progenitors was assessed at 28 hpf by in situ hybridization to *hhx*. The following pharmacologic agents were used: sulprostone (Ep1 + Ep3 agonist), Ono 248 (Ep3 agonist), butaprost (Ep2 agonist), Ono 259 (Ep2 agonist), and Ono 329 (Ep4 agonist). (*Significant versus control by χ^2 test, $p < 0.05$; $n > 30$ for each condition.)

(F) Expression of *ep2a* and *ep4a* at 72 hpf visualized by in situ hybridization. *ep4a* but not *ep2a* expression at 72 hpf corresponds to the emerging liver bud.

(G) Size distribution of pancreas or liver organs following exposure to pharmacologic agonists or antagonists of various PGE₂ receptors from 48 to 96 hpf. Impact on pancreas outgrowth was assessed at 96 hpf by in situ hybridization to *trypsin*, whereas impact on liver outgrowth was assessed at 96 hpf by in situ hybridization to *lfabp*. (*Significant versus control by χ^2 test, $p < 0.05$; $n > 30$ for each condition.)

See also Figure S6.

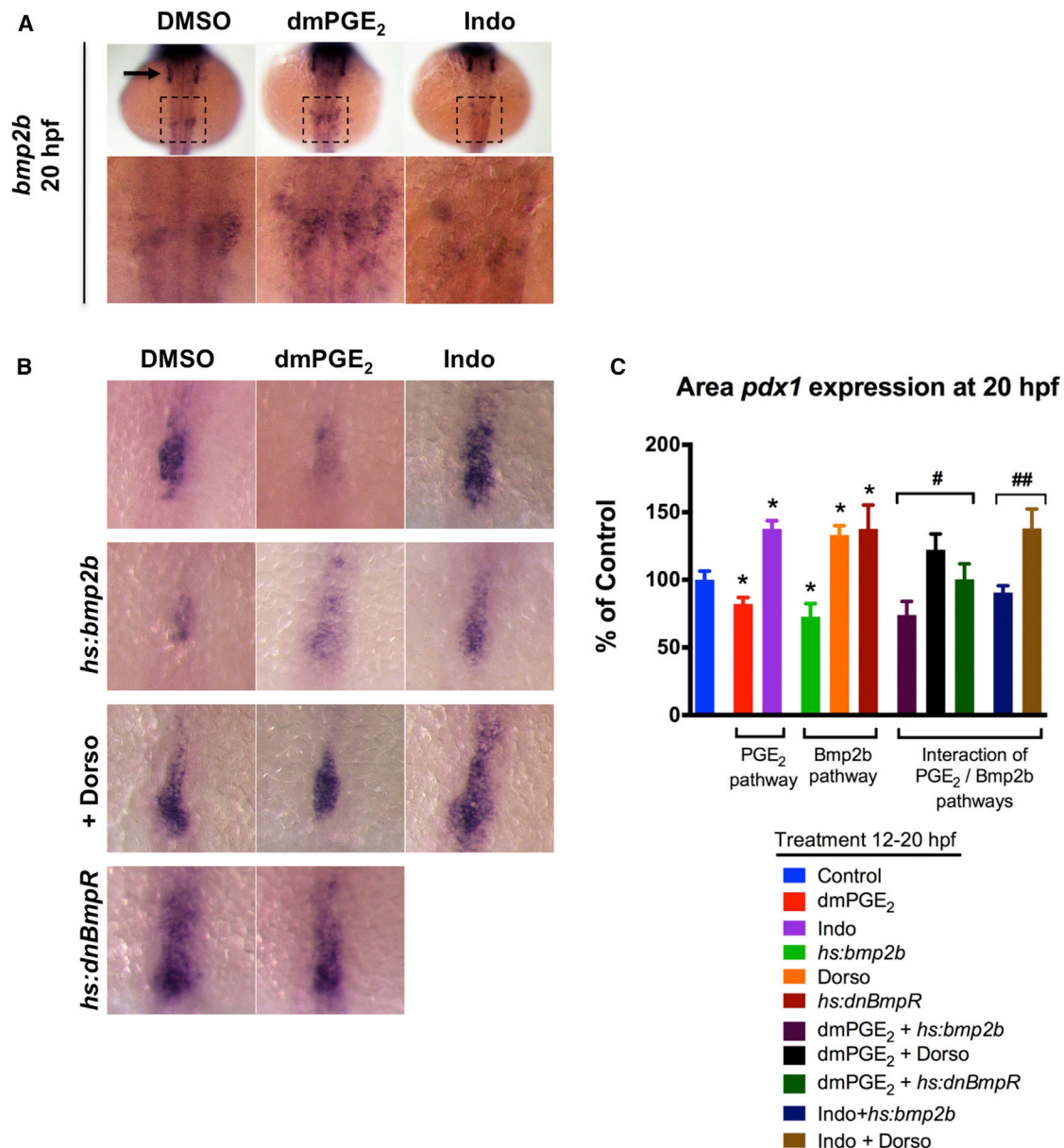


Figure 6. PGE₂ Interacts with the *bmp2b* Pathway to Regulate Liver versus Pancreas Specification

(A) Modulation of PGE₂ levels from 12 to 20 hpf impacts expression of *bmp2b* in the lateral plate mesoderm as shown by in situ hybridization at 20 hpf. dmPGE₂ increases *bmp2b* expression ($n = 15/19$ embryos), whereas Indo decreases *bmp2b* expression ($n = 14/21$ embryos). Lower: magnified view of the area in the dashed square. *bmp2b* is also expressed in the otic vesicles (arrow).

(B and C) Size of pancreas-specified population marked by *pdx1* is impacted by modulation of the PGE₂ or *bmp2b* pathways. Representative views are shown in (B), and quantification is shown in (C). dmPGE₂ or heat-shock activation of *bmp2b* diminishes whereas Indo, dorsomorphin (Dorso), or heat-shock activation of *dnBmpR* promotes the pancreas progenitor population. The impact of dmPGE₂ is blunted by Dorso or *dnBmpR*, and the impact of Indo is blunted by heat-shock activation of *bmp2b*. (Data are represented as mean \pm SEM; $n \geq 10$ per condition; t test comparisons: * $p < 0.05$ comparing treatment versus control; #no significant difference comparing with versus without dmPGE₂; ##no significant difference comparing with versus without Indo.)

of PGE₂ promote a liver specification program, whereas lower levels or absence of PGE₂ promotes a pancreas specification program. A similar activity gradient regulating liver versus pancreas specification has been postulated of *bmp2b* (Chung et al., 2008), and indeed we find evidence for an interaction between the PGE₂ and *bmp2b* pathways. How different levels of PGE₂ are transduced into a liver versus pancreas cell fate, and how

this activity gradient interacts with other signals known to regulate liver and pancreas specification, remains to be elucidated.

Dynamic Roles for PGE₂ in Different Developmental Contexts

Our findings demonstrate that the role of PGE₂ activity in the context of liver and pancreas development changes over time.

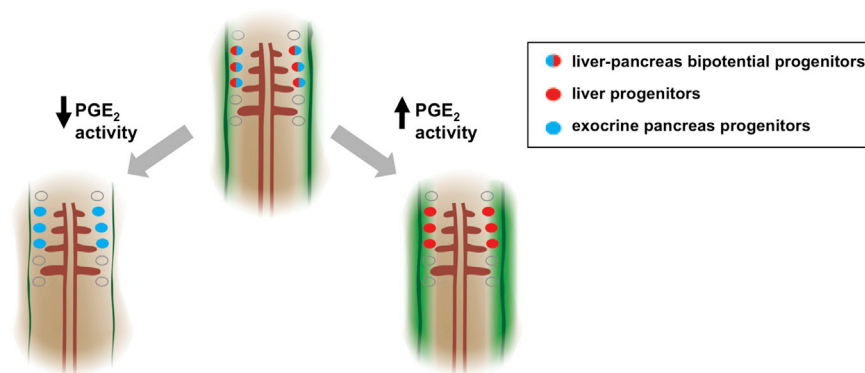


Figure 7. Model for the Role of PGE₂ Activity in Liver versus Pancreas Endoderm Specification

A model for PGE₂ regulation of endodermal specification into liver or pancreas progenitors. Bipotential endodermal cells capable of both a liver and an exocrine pancreas fate (red/blue cells) have been found between somites 1 and 3. Levels of PGE₂ (green gradient) may form a gradient with highest activity closest to the two bilateral stripes of *cox2a* (solid green bands), the rate-limiting enzyme in PGE₂ synthesis. Cells closest to the highest levels of PGE₂ are more likely to acquire a liver fate (red cells), whereas cells farther from the source of PGE₂ acquire an exocrine pancreas fate (blue cells). Augmenting levels of PGE₂ promotes a liver fate at the cost of a pancreas fate, whereas decreasing levels of PGE₂ promotes a pancreas fate at the cost of a liver fate.

This is a fundamental property of other highly conserved signaling pathways that regulate liver and pancreas development. Shifting roles in liver and pancreas development have been demonstrated for Wnt, Shh, and Bmp signaling pathways, and these changes can occur in just a few hours (Goessling et al., 2008; Wandzioch and Zaret, 2009; Zaret and Grompe, 2008). Similarly, in this study, we find a time frame during early somitogenesis in which PGE₂ activity regulates liver versus pancreas specification of gut endoderm and a later phase in which PGE₂ activity promotes the expansion and outgrowth of both liver and pancreas buds. A changing role for PGE₂ might in part be governed by exquisite spatial and temporal regulation of receptor expression in different contexts. We find that *ep2a* and *ep4a* are differentially patterned in different developmental contexts. Moreover, consistent with their distinct expression, we demonstrate receptor-specific effects of blocking *ep2a* or *ep4a*, and find that *ep2a* has a critical role in regulating liver- versus pancreas-fate decisions, whereas *ep4a* has a role in regulating outgrowth of the liver and pancreas buds. In conjunction with carefully regulated production, the regulation of receptor expression to dictate shifting roles of secreted factors might be a common theme in development. Delineating the role of prostaglandins at specific stages of liver and pancreas organogenesis might help inform how we tailor stem cell differentiation for therapy.

The Role of PGE₂ in Gastrointestinal Regeneration and Cancer

A second fundamental property of signaling pathways that regulate solid organ development is their importance in the context of regeneration and cancer (Goessling et al., 2008; Rhim and Stanger, 2010). Indeed, in this study, we show that modulating prostaglandin activity influences liver regeneration after adult hepatectomy. These findings parallel our observations that genetically or chemically diminishing prostaglandin activity abrogates outgrowth of the embryonic liver bud. Furthermore, consistent with our findings that excess prostaglandin activity promotes growth of the embryonic pancreas bud and primordial intestine marked by *ifabp*, dysregulation of the prostaglandin pathway is a key feature of both pancreatic and colon cancer, and inhibitors of COX synthetic enzymes have long been known to reduce the occurrence and death from cancers of the colon, lungs, and other

endodermal derivatives (Rothwell et al., 2011; Wang and Dubois, 2010). Therefore, although prostaglandins have traditionally been thought to promote cancer by augmenting inflammation and angiogenesis, the findings presented in this study provide an ontogenic context for investigating the role of prostaglandin activity in gastrointestinal cancer and regeneration.

A New Understanding of PGE₂ Function

A dynamic array of protein signaling factors has been implicated in regulating specification of gut endoderm (Zaret and Grompe, 2008). Our findings place PGE₂ among these canonical signaling pathways, and thereby challenge long-standing notions of prostaglandin function. We demonstrate that, much like canonical protein signaling pathways, the PGE₂ pathway exhibits exquisite spatial and temporal patterning of its synthetic enzymes and receptors, changing roles at different times in organogenesis, and a role in adult organ homeostasis. From an evolutionary perspective, prostaglandins have been found in prokaryotes and plants, and thus predate the protein signaling pathways that regulate development of metazoans (Richards and Degnan, 2009). We postulate that prostaglandins offer unique ancestral advantages compared to protein signaling pathways. Prostaglandins are synthesized de novo from metabolism of membrane-derived arachidonic acid, and therefore do not have the storage and energy requirements of protein signaling pathways. Additionally, prostaglandins are thought to have an evanescent half-life (Funk, 2001), and therefore might allow more temporally and spatially accurate signaling between cells. The specification of endoderm into liver or pancreas fates, in which regulatory signals must act over the span of a few cells and in a small time window, is exemplary of such a sensitive and critical developmental process. Our findings open the door to the discovery of additional developmental programs regulated by prostaglandins and might offer new therapeutic avenues for tissue repair and cancer treatment.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish were maintained according to Institutional Animal Care and Use Committee protocols. Transgenic lines *ifabp:GFP*, *lf:dsRed;elastase:GFP*, *gut:GFP*, *trypsin:GFP*, *hs:bmp2b*, and *hs:dnBmpR* were described previously (Chung et al., 2008; Goessling et al., 2008; Korzh et al., 2008). Heat shock of

hs:bmp2b fish or *hs:dnBmpR* fish at 12 hpf was performed at 37°C or 40°C for 30 min as previously described (Chung et al., 2008).

Embryonic Zebrafish Experiments

Embryos were exposed to pharmacologic agents at the time points described at a concentration of 10 μ M except for 30 μ M dorsomorphin, 0.5 μ M H89, and 0.5 μ M forskolin. DMSO carrier content was 0.1%. Embryos were processed for in situ hybridization using standard protocols (<http://zfin.org/ZFIN/Methods/ThisseProtocol.html>). Morpholino (GeneTools) knockdown was performed as previously described (North et al., 2007). Embryos were processed for whole-mount immunohistochemistry using standard protocols and antibodies against pHH3 (Millipore; titer 1:500) and GFP (Millipore; titer 1:300). For section immunohistochemistry, hematoxylin and eosin staining was performed on alternate sections using standard techniques as previously described (Goessling et al., 2008). Antibodies to proliferating cell nuclear antigen (AnaSpec; titer 1:200) were visualized by 3,3'-diaminobenzidine and counterstained with methyl green. Apoptosis was visualized using the ApopTag kit (Chemicon).

Adult Zebrafish Experiments

Liver resection was performed and regeneration was evaluated as previously described (Goessling et al., 2008). Adult zebrafish were fixed with paraformaldehyde, paraffin embedded, and cut in 10 μ m sections for histological analysis. Hematoxylin and eosin staining was performed on alternate sections using standard techniques as previously described (Goessling et al., 2008).

Quantification of Liver or Pancreas Size

Liver or pancreas size was quantified by FACS of GFP⁺ cells in fluorescent reporter fish, confocal microscopy, and volumetric analysis, and measurement of the area of marker expression was visualized by in situ hybridization using ImageJ (National Institutes of Health). Volumetric analysis incorporated volume and average fluorescence intensity of 3D confocal stacks using ImageJ.

Flow Cytometry Analysis

Whole fluorescent embryos were manually dissociated in 0.25% trypsin for 20 min and then analyzed for % GFP fluorescence by flow cytometry. At least 100,000 cells were analyzed per sample (n = 10–20 per treatment).

Quantitative PCR

Quantitative PCR was performed using primer sets for *pdx1*, *ep2a*, and *ep4a*. RNA was extracted and RT-PCR was performed as previously described (Goessling et al., 2009).

Mouse Embryonic Stem Cell Differentiation, Treatment, and Immunohistochemistry

Mouse embryonic stem cell culture and stepwise differentiation into endoderm and then bipotential hepatopancreatic progenitors were performed according to previously published protocols (Sherwood et al., 2011) as schematized in Figure S4 and detailed in Supplemental Experimental Procedures.

Microarray

Bipotential hepatopancreatic progenitors were derived from mouse embryonic stem cells as described, processed for microarray using the Illumina TotalPrep RNA Amplification Kit (Ambion), and hybridized to MouseRef-8 v2.0 chips (Illumina). Microarray values were compared with previously obtained microarray data (R. Sherwood, personal communication) using GenomeStudio software (Illumina).

Statistical Analysis

Pooled data were calculated as mean \pm SE, with the number of repeats as indicated. Pairwise comparison was performed by t test, multiple comparisons by ANOVA, and distribution comparisons by χ^2 test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2014.01.006>.

ACKNOWLEDGMENTS

This work was supported by the National Pancreas Foundation and National Institutes of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant T32 (S.N.) and by NIH NIDDK grants R03DK085445 and R01DK090311 (W.G.) and K01DK080226 (T.E.N.), the Harvard Stem Cell Institute (W.G. and T.E.N.), and the Pew Charitable Trusts (W.G.).

Received: May 17, 2013

Revised: December 18, 2013

Accepted: January 10, 2014

Published: February 13, 2014

REFERENCES

- Burke, Z., and Oliver, G. (2002). Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. *Mech. Dev.* 118, 147–155.
- Chung, W.S., Shin, C.H., and Stainier, D.Y. (2008). Bmp2 signaling regulates the hepatic versus pancreatic fate decision. *Dev. Cell* 15, 738–748.
- Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 129, 1819–1828.
- Deutsch, G., Jung, J., Zheng, M., Lora, J., and Zaret, K.S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* 128, 871–881.
- Funk, C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871–1875.
- Garnaas, M.K., Cutting, C.C., Meyers, A., Kelsey, P.B., Jr., Harris, J.M., North, T.E., and Goessling, W. (2012). Rargb regulates organ laterality in a zebrafish model of right atrial isomerism. *Dev. Biol.* 372, 178–189.
- Goessling, W., North, T.E., Lord, A.M., Ceol, C., Lee, S., Weidinger, G., Bourque, C., Strijbosch, R., Haramis, A.P., Puder, M., et al. (2008). APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. *Dev. Biol.* 320, 161–174.
- Goessling, W., North, T.E., Loewer, S., Lord, A.M., Lee, S., Stoick-Cooper, C.L., Weidinger, G., Puder, M., Daley, G.Q., Moon, R.T., and Zon, L.I. (2009). Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* 136, 1136–1147.
- Haumaitre, C., Barbacci, E., Jenny, M., Ott, M.O., Gradwohl, G., and Cereghini, S. (2005). Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. *Proc. Natl. Acad. Sci. USA* 102, 1490–1495.
- Jacquemin, P., Lemaigre, F.P., and Rousseau, G.G. (2003). The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. *Dev. Biol.* 258, 105–116.
- Korzh, S., Pan, X., Garcia-Lecea, M., Winata, C.L., Pan, X., Wohland, T., Korzh, V., and Gong, Z. (2008). Requirement of vasculogenesis and blood circulation in late stages of liver growth in zebrafish. *BMC Dev. Biol.* 8, 84.
- Lokmane, L., Haumaitre, C., Garcia-Villalba, P., Anselme, I., Schneider-Maunoury, S., and Cereghini, S. (2008). Crucial role of vHNF1 in vertebrate hepatic specification. *Development* 135, 2777–2786.
- Miki, R., Yoshida, T., Murata, K., Oki, S., Kume, K., and Kume, S. (2012). Fate maps of ventral and dorsal pancreatic progenitor cells in early somite stage mouse embryos. *Mech. Dev.* 128, 597–609.
- Müller, P., Rogers, K.W., Jordan, B.M., Lee, J.S., Robson, D., Ramanathan, S., and Schier, A.F. (2012). Differential diffusivity of Nodal and Lefty underlies a reaction-diffusion patterning system. *Science* 336, 721–724.
- North, T.E., Goessling, W., Walkley, C.R., Lengerke, C., Kopani, K.R., Lord, A.M., Weber, G.J., Bowman, T.V., Jang, I.H., Grosser, T., et al. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* 447, 1007–1011.

- Rausa, F., Samadani, U., Ye, H., Lim, L., Fletcher, C.F., Jenkins, N.A., Copeland, N.G., and Costa, R.H. (1997). The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 β in the developing murine liver and pancreas. *Dev. Biol.* 192, 228–246.
- Rhim, A.D., and Stanger, B.Z. (2010). Molecular biology of pancreatic ductal adenocarcinoma progression: aberrant activation of developmental pathways. *Prog. Mol. Biol. Transl. Sci.* 97, 41–78.
- Richards, G.S., and Degnan, B.M. (2009). The dawn of developmental signaling in the metazoa. *Cold Spring Harb. Symp. Quant. Biol.* 74, 81–90.
- Rogers, K.W., and Schier, A.F. (2011). Morphogen gradients: from generation to interpretation. *Annu. Rev. Cell Dev. Biol.* 27, 377–407.
- Rossi, J.M., Dunn, N.R., Hogan, B.L., and Zaret, K.S. (2001). Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev.* 15, 1998–2009.
- Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P., and Meade, T.W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 377, 31–41.
- Schuster, V.L. (2002). Prostaglandin transport. *Prostaglandins Other Lipid Mediat.* 68–69, 633–647.
- Sherwood, R.I., Maehr, R., Mazzoni, E.O., and Melton, D.A. (2011). Wnt signaling specifies and patterns intestinal endoderm. *Mech. Dev.* 128, 387–400.
- Shimozono, S., Imura, T., Kitaguchi, T., Higashijima, S., and Miyawaki, A. (2013). Visualization of an endogenous retinoic acid gradient across embryonic development. *Nature* 496, 363–366.
- Spence, J.R., Lange, A.W., Lin, S.C., Kaestner, K.H., Lowy, A.M., Kim, I., Whitsett, J.A., and Wells, J.M. (2009). Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. *Dev. Cell* 17, 62–74.
- Tremblay, K.D., and Zaret, K.S. (2005). Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev. Biol.* 280, 87–99.
- Wandzioch, E., and Zaret, K.S. (2009). Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* 324, 1707–1710.
- Wang, D., and Dubois, R.N. (2010). Eicosanoids and cancer. *Nat. Rev. Cancer* 10, 181–193.
- Zaret, K.S., and Grompe, M. (2008). Generation and regeneration of cells of the liver and pancreas. *Science* 322, 1490–1494.